The Chronic Idiopathic Neutropenia Syndrome: Correlation of Clinical Features With In Vitro Parameters of Granulocytopoiesis

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The natural history and possible pathogenetic mechanisms underlying the chronic idiopathic neutropenia syndrome (CINS) were evaluated in 41 prospectively studied adult patients. The low neutrophil counts in 90% of the patients remained stable during the 6.1-yr median period of observation. Infectious complications occurred in 27% of the patients, with an enhanced susceptibility to infection being found in individuals with ≤500 neutrophils/cu mm. Agar-medium marrow culture techniques were utilized to assess growth potential of granulocytic precursors (granulocytic colony-forming cells, CFU-GM) and intramedullary cellular production of granulocytic colony-stimulating activity (CSA). In patients with <1000 neutrophils/cu mm adequate responsive marrow CFU-GM were present, as the incidence of these cells and their proportion in DNA synthesis were increased. In contrast, levels of CSA were decreased. Values of CSA reflect stimulatory influences of the marrow microenvironment, and the low levels may contribute to defective granulocytic maturation found in these neutropenic individuals. Antineutrophil opsonizing antibodies were present in serum of only 3 of 19 (16%) subjects tested. Our patients, who remain clinically stable, had a normal proportion of light density marrow CFU-GM, which helps to distinguish them from other groups of neutropenic patients with the potential for progression to leukemia or other myeloproliferative disorders.

CHRONIC IDIOPATHIC NEUTROPENIA provides a clinical setting for evaluating abnormalities of granulocytic regulation. Patients with this hematologic abnormality have a refractory neutropenia despite lack of splenomegaly, history of exposure to toxic drugs, or evidence of systemic disease. The family history is generally negative for neutropenia. Morphological examination of the marrow is essentially normal and does not clearly explain the neutropenia. Disparate pathophysiological mechanisms, including decreased production, enhanced peripheral removal, and excessive margination of neutrophils, have been found in individuals with this constellation of clinical features. Because the neutropenias have potentially heterogeneous etiologies, we have utilized the descriptive term chronic idiopathic neutropenia syndrome (CINS) to describe patients with these clinical findings.

Development of in vitro marrow culture techniques has permitted assessment of granulocytic progenitor cells (CFU-GM) and the humoral stimulatory substance necessary for growth of these cells in vitro, termed colony-stimulating activity (CSA). Evidence is accumulating regarding the possible physiologic relevance of these parameters of granulocytopoiesis. The cell cycle status of CFU-GM reflects the responsiveness for regeneration of myeloid precursor cells. The density distribution profile of these granulocytic precursors has provided a qualitative physical marker helping to distinguish normal from leukemic progenitor cells. Recent studies have shown the presence of cells capable of producing CSA within the microenvironment of the bone marrow (CSA) in mouse and man, suggesting that such local influences of granulocytopoiesis may be critical for the proliferation and maturation of granulocytic cells.

In order to determine possible pathogenetic mechanisms underlying the CINS, we have evaluated these in vitro parameters of granulocytopoiesis in these patients. We have prospectively correlated these indices with clinical courses in 41 adult patients with this disorder to determine whether factors predictive of neutrophil levels, natural history, or infectious complications could be defined. We specifically examined the relationship between neutrophil levels, clinical courses, and in vitro marrow culture data by segregating these patients into subgroups with relative degrees of neutropenia.

MATERIALS AND METHODS

Marrow Colony-Forming Capacity

Our techniques of performing assays for granulocytic colony-forming capacity by marrow CFU-GM have previously been described. Human marrow cells obtained by aspiration were heparinized, sedimented in 3% dextran, washed, and 2 x 10^7 cells were plated in a 1-ml 0.3% agar-medium (McCoy's modified 5A medium with 15% fetal calf serum -- FCS) layer over a human leukocyte feeder in 1 ml of 0.5% agar-medium. The plates were allowed to gel and were incubated at 37°C in a humidified air-CO_2 incubator for 7–10 days. Colonies consisted of groups of >50 cells and were morphologically identified as granulocytic and monocytic

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cells at various stages of differentiation. Normal CFU-GM values were 26 ± 7.5 colonies/10^5 marrow cells plated (mean ± SD).

**CFU-GM in DNA Synthesis**

To determine the proportion of CFU-GM in DNA synthesis, thymidine suicide studies were performed as previously described.7 Marrow suspensions from patients were prepared in serum-free modified McCoy’s medium, which contains no thymidine. The cell suspension was placed in a centrifuge with medium containing ^3H-thymidine (Schwarz-Mann) in a final concentration of 12.5 μCi/ml. The specific activity of the radioisotope was 16 Ci/mmole. A control tube, lacking ^3H-thymidine, was studied in parallel. After 20 min of incubation at 37°C, the cellular uptake of the ^3H-thymidine was stopped by the addition of 30 ml of ice-cold serum-containing medium with 100 μg/ml (0.4 mM) of unlabeled thymidine. The cell suspensions were washed, and replicate dishes were plated in medium containing 10 μg/ml unlabeled thymidine in an agar layer overlying a normal human leukocyte feeder layer. After a standard incubation for 7–10 days, colony number and size and cell morphology were evaluated. Normal values of the proportion of CFU-GM in DNA synthesis were 35±3%. The difference in the number of colonies formed from the cell suspension exposed to ^3H-thymidine relative to the number formed in the unexposed control suspension indicated the proportion of granulocytic progenitor cells that were in S phase during exposure. Only those cells in the S phase of the cell cycle had their proliferative activity inhibited. Thus, it should be understood that the percentage expressed represents an underestimate of the actual proportion of cells in active cycle.

**Marrow Cell CSA Provision**

Adherent marrow cells were obtained as previously described.12 Marrow cells were centrifuged on a Hypaque-Ficoll gradient (density 1.077 g/cc) for 20 min at 800 g. The bouyant cells were washed in McCoy’s medium, and 10^7 cells in 2 ml medium containing 7.5% FCS were placed in 35 x 10 mm tissue culture dishes for 60 min at 37°C. Nonadherent cells were removed by rinsing, and the (bouyant) adherent cells were incubated in 1 ml McCoy’s medium with 15% FCS and 0.5 mM 2-mercaptoethanol for 7 days at 37°C in an air–CO_2 incubator. Nonadherent cells did not produce CSA. After incubation, the supernatant-conditioned medium was harvested and stored at -20°C until use. The conditioned media were tested for CSA by determining colony formation in single agar layer culture 7–10 days after plating 0.15 ml serial aqueous dilutions of the media with 0.2 x 10^7 bouyant nonadherent normal marrow target cells. Control plates containing nonadherent bouyant marrow target cells without a CSA source were included in all experiments and consistently showed no colony formation. The number of adherent cells was calculated by determining the difference between the total number of cells plated and the nonadherent cells recovered after washing the plates. Direct counting of the adherent cells in measured areas of the tissue culture dishes generally confirmed the calculated adherent cell values in representative experiments. The CSA in test conditioned medium were compared to that present in a stable human mononuclear leukocyte-conditioned medium CSA standard. Dilution curves of CSA (colonies formed) permitted quantitative estimates of effective CSA concentrations and were analyzed by previously described curve-fitting computer programs.13 Marrow CSA provision from 16 normal subjects was 26 U/ml (geometric mean, as data were positively skewed, 95% confidence limits 18–38 U/ml).

**Albumin Density Separation Procedures**

Our procedures for performing the bovine serum albumin continuous gradient equilibrium centrifugation technique and the neutral density (density cut) procedure for evaluating density distribution patterns of marrow cells have been previously described.8,9 For continuous gradients, linear albumin gradients were generated in the density range 1.055–1.075 g/cc and contained 10^–10^6 marrow cells. Gradients were centrifuged for 40 min at 5000 g at 4°C. Fractions were collected at 4°C by upward displacement, suspended in medium, and plated for CFU-GM determination. Refractive index (Bausch and Lomb Abbé 3L Refractometer) was employed for evaluating fraction density. Distribution profiles were calculated as CSA per density increment against fraction density. For the neutral density procedures, the bone marrow cells were dispersed in albumin (density 1.062 g/cc) and centrifuged for 10 min at 4000 g at 4°C. Cells in suspension and pellets were recovered separately and diluted in medium, centrifuged, resuspended, and plated in agar for determination of CFU-GM. Normal values for the proportion of light density CFU-GM were 2.8% ± 2.6% (mean ± SD).8

**Opsonizing Antibodies**

The methods used to assay antineutrophil antibodies have been described in detail elsewhere.14 Serum specimens, obtained from a sequential group of these patients, were heat inactivated at 56°C for 30 min. Neutrophils attempt to ingest other neutrophils sensitized with antibody. The attempt at ingestion activates the oxidative metabolism of the ingesting (inducer) leukocytes and can be monitored by measurement of glucose oxidation. Human peripheral blood neutrophils (test cells) were incubated with 133 μl of test serum per 10^6 neutrophils at 25°C for 30 min in the presence of 10 mM 2-deoxyglucose to inhibit the metabolism of the test cells. Washed cell pellets were suspended in modified Krebs-Ringer phosphate buffer at a final concentration of 9 x 10^6 cells per ml with 2 x 10^4 leukocytes (indicator cells) containing 0.5 μCi of ^14C-glucose and 1 mM carrier glucose. The initial rate of glucose metabolism was determined by scintillation spectrophotometry of evolved ^14CO_2. A test was considered positive if the glucose oxidation rates exceeded 2 SD above the control mean.

Serum muramidase values were evaluated by previously described methods as a measure of the turnover of myeloid mass,15 with normal values being 15–45 μg/ml. Leukocyte alkaline phosphatase (LAP) values were determined by previously described methods8 as a measure of secondary granule content of the neutrophils, with normal LAP scores being 50–150/100 neutrophils.

**Patient Selection**

The 41 patients (38 white, 3 black) studied at the Stanford University and Palo Alto Veterans Administration Medical Centers had chronic neutropenia (~1800 neutrophils/μl mm) for at least 1.5 yr with no splenomegaly or exposure to toxic drugs. Clinical features are denoted in Table 1. The blood counts given are those obtained at the time of marrow culture. Serial blood counts, initially at weekly intervals, then monthly for at least 3 mo, and at subsequent follow-up, indicated relative constancy of these values (except for 6 patients detailed below). The patients had no clinical or serologic evidence of systemic diseases (particularly connective tissue disorders). A lack of family history for neutropenia was documented only by history in the majority of cases, although blood counts were obtained from family members of several patients. Thus, we cannot exclude a familial basis for CINS in some patients. Examination of marrow aspirates and clot sections generally showed normocellularity with normal myeloid maturation. Twenty-seven percent of the
patients had a decrease in late myeloid cells (<25% neutrophils and bands)\(^a\). The morphology of the erythroid and megakaryocytic cells was normal, as were the patients' hematocrits and platelet counts, except for four patients with mild anemia and one patient with thrombocytopenia.

Statistical analyses were performed using the Wilcoxon two-sample two-tailed rank test, the chi-square test with Yates' correction, and the Pearson correlation coefficient. Differences were considered statistically significant at \(p < 0.05\).

**RESULTS**

Table 1 provides the composite clinical and in vitro marrow culture data of our 41 patients, ranked in
ascending order of their neutrophil counts. The median age of the patients was 39 yr, and 68% of the patients were female. The patients' diseases were followed for a median period of 6.1 yr (mean ± SE, 6.8 ± 0.7 yr), including 3 yr following in vitro culture. All patients were still living at the time of the last evaluation. Neutrophil counts of these patients were 824 ± 82/cu mm and monocytes were 328 ± 30/cu mm. Neutrophil counts were <1000/cu mm in 59% and ≤500/cu mm in 39% of patients. Twenty-five percent of the patients had monocytopenia (<200 monocytes/cu mm), and none had monocytosis. No correlation was noted between neutrophil and monocyte counts. However, patients with a decreased proportion of late marrow myeloid cells (polys and bands) had significantly lower peripheral neutrophil counts than the remaining patients (p < 0.02). Seventy-two percent of patients with decreased late myeloid cells had ≤500 neutrophils/cu mm, whereas such low levels of neutrophils were present in only 27% of patients with normal myeloid differential counts of marrow. Fifty percent of patients with ≤500 neutrophils/cu mm had a decreased percentage of late myeloid cells versus 12% of patients with higher neutrophil counts (p < 0.02). Throughout the follow-up period, stable low neutrophil counts persisted in 37 patients (90%), and 4 patients (initially <1000 neutrophils/cu mm) had spontaneous return of neutrophil counts to normal. The very low neutrophil counts of 2 additional patients (nos. 1 and 2) became normal after therapy with corticosteroids (the only patients so treated; one patient had opsonizing antibodies).

Sixteen percent of patients (3 of 19) had opsonizing antibodies demonstrable in serum. Serum muramidase levels were normal in 23 of 30 patients (77%) and low in 5 patients (17%). The two patients with increased muramidase values had positive opsonizing antibodies. Two of three patients with serum opsonizing antibodies had increased serum muramidase levels, whereas all of the 13 patients tested with negative opsonizing antibodies had normal to low values of muramidase (p < 0.05). Decreased values of LAP were present in 7 of 16 patients (44%). No differences were noted in these parameters relative to the patients' neutrophil levels.

Figure 1 denotes the incidence and cell-cycle status of marrow CFU-GM, the proportion of light density CFU-GM, and the production of marrow adherent cell CSA (CSA<sub>BM</sub>) in patients with the CINS. A wide range of marrow incidence of CFU-GM was present, with increased mean values of 39 ± 3.8 colonies/10<sup>5</sup> marrow cells (p < 0.01). Eighty-six percent (35 of 41) of these values were normal or increased. The proportion of CFU-GM in DNA synthesis was normal for the group of patients tested (35% ± 3.8%, mean ± SE). However, as shown in Table 1, the proportion of CFU-GM in DNA synthesis (43% ± 5.9%) was increased for patients with <1000 neutrophils/cu mm compared to 23.6% ± 5% for individuals with higher neutrophil values (p < 0.05). A generally inverse, although statistically insignificant, relationship (as determined by linear regression analysis, correlation coefficient r = −0.51) was found between CFU-GM in DNA synthesis and peripheral neutrophil counts. A statistically significant relationship was also not found between marrow morphology and the proportion of CFU-GM in DNA synthesis. Repeat marrow culture studies in 4 clinically stable patients showed relative constancy of CFU-GM over a period of 2–17 mo. Initial and repeat values of CFU-GM/10<sup>5</sup> marrow cells from patients 2, 7, 8, and 38 were 52 and 48, 38 and 40, 60 and 72, 20 and 24, respectively. In 6 patients, a normal proportion of light density (i.e., <1.062 g/cc) CFU-GM was present, 2.9% ± 1.7%. The complete density gradient analysis performed in patient no. 5 was also normal (Fig. 2), in contrast to the density distribution pattern of CFU-GM from a representative patient with chronic myeloid leukemia (see references 8 and 13). Values of CSA<sub>BM</sub> were normal or decreased in all 12 patients tested, and as a group, these values were significantly lower than normal, with a geometric mean value of 10 U/ml conditioned medium (95% confidence limits 5–20 U/ml) (p < 0.05). These low CSA<sub>BM</sub> values are not related to the patients' neutrophil counts (Table 1) or to a subnormal number of adherent marrow cells, since 9.3% ± 2.5% (9.3 × 10<sup>5</sup> cells) were adherent. Normal values for this parameter are 8.6% ± 3.2%, and plateau levels of CSA are produced by this number of cells. Sera from 5 patients tested against autologous or heterologous marrow showed no...
Evidence of inhibition of CFU-GM. CSA was also not detected in these serum samples when nonadherent buoyant human marrow cells were utilized as target cells.

We determined whether predictive clinical features or in vitro parameters correlated with infectious complications or subsequent normalization of neutrophil counts. Mild to moderate bacterial infections (particularly sinusitis, upper respiratory infections, or cellulitis) were notable features in 11 of 41 patients (27%). The patients with infections had significantly lower neutrophil counts than those 30 patients without these problems, 437 ± 125/cu mm versus 967 ± 90/cu mm (p < 0.004). The 39% of patients with ≤500 neutrophils/cu mm had a higher incidence of infections than the remaining patients (56% versus 8%, p < 0.002). Eighty-one percent of patients with infections had ≤500 neutrophils/cu mm compared to 23% of patient lacking these complications (p < 0.002). As described above, significant correlation was found also between the proportion of marrow CFU-GM in DNA synthesis and peripheral neutrophil counts. No other significant associations were demonstrated between the in vitro parameters of granulocytopoiesis assessed, clinical features, normalization of neutrophil counts, or infectious complications.

DISCUSSION

Patients with the chronic idiopathic neutropenia syndrome (CINS) present with a similar constellation of clinical features, but a unifying pathogenic mechanism to categorize them as having a homogeneous disease entity has not been demonstrated. The major purposes of our study were to assess the natural history and to define possible pathogenetic and prognostic factors of the CINS. Our analyses indicated that the levels of neutrophils in the vast majority of these patients (90%) remained unchanged over a 6.1-yr median period of follow-up. None of these patients' neutropenias evolved into other hematologic disorders. Specifically, this abnormality did not presage leukemia.

Prior studies have demonstrated a normal total-body granulocyte pool but enhanced margination of neutrophils in most neutropenic patients with >1000 neutrophils/cu mm. Thus, the patients were ranked according to their circulating neutrophil counts to determine whether clinical features or parameters of granulocytopoiesis correlated with these values. Mild to moderate infectious complications occurred in 27% of the patients. These problems were significantly associated with neutrophil levels ≤500/cu mm and occurred in 81% of these patients. A significantly higher proportion of patients with diminished late myeloid cells in marrow had peripheral neutrophil counts ≤500/cu mm. Prior studies have also shown a similar relationship in patients with chronic neutropenia, equating this morphological finding with a decreased marrow granulocytic reserve.

In vitro marrow growth studies assessing patterns of granulocytopoiesis were performed prospectively to determine the possible clinical utility and pathogenetic implications of these parameters in patients with the CINS (Fig. 1). These indices of granulocytopoiesis indicated a variable though significantly increased incidence of CFU-GM. Prior studies have suggested that the proportion of CFU-GM in DNA synthesis is a more accurate reflection of granulocytopoietic responsiveness than the incidence of CFU-GM. The former measurement would not be affected by dilution by peripheral blood, marrow differential counts, or cell processing. Evaluation of CFU-GM incidence, which denotes the concentration rather than the quantitation of CFU-GM, has these drawbacks, which limit the utility of this measurement. As a group, the proportion of CFU-GM in DNA synthesis was normal. Patients with neutrophil counts <1000/cu mm demonstrated significantly elevated proportions of CFU-GM in DNA synthesis in contrast to these neutropenic patients having higher levels of neutrophils. This
suggests that the myeloid precursor cells of patients with <1000 neutrophils/cu mm may be appropriately responsive to demand for new cells. Combined with finding no decrease of the incidence of CFU-GM in marrow in 86% of cases, these data indicate that a quantitative lack of responsive granulocyte precursors is not the cause of this syndrome. In patients with 1000–1800 neutrophils/cu mm, lack of an increased proportion of CFU-GM in DNA synthesis may reflect suboptimal response of granulocytopoiesis. Alternatively, as mentioned above, this finding may relate to a suboptimal response of granulocytosis. Alterna-

tion of human marrow cells, in contrast to murine

CSABM

ry.6"2 Significantly decreased values of CSA

are needed for the in vitro stimula-
tion of human marrow cells, in contrast to murine marrow where urine and serum are also stimulato-

CSA-enhancing substances, inhibitory for

myeloid precursors, were not found in the few patients so tested.

Cellular sources of CSA (i.e., medium conditioned by cells in culture) are needed for the in vitro stimula-
tion of bone marrow cells. Thus, this abnormality of limiting amounts of CSA may contribute to defective myeloid maturation in the CINS. Defective myeloid maturation and ineffective granulocytopoiesis have previously been shown in some patients with this syndrome.21,22 The continual presence of CSA is needed for maturation of cells comprising myeloid colonies in vitro.23,24 The low values of CSA in marrow where urine and serum are also stimulato-

patients was normal, using both the density cut and continuous density gradient procedures. Thus, this parameter appears to be a useful adjunct to marrow morphology for distinguishing this group of patients who have remained clinically stable from neutropenic patients with the preleukemic syndrome (hemopoeitic dysplasia) or other prodromal myeloproliferative disorders who may otherwise present with clinical similarities.9,27

Sixteen percent of patients tested had opsonizing antibodies in serum, suggesting that enhanced peripheral removal of neutrophils related to this mechanism could have contributed to the neutropenia in only a minority of subjects. Recent in vivo leukokinetic studies have also indicated normal peripheral blood neutrophil survival and a low incidence of opsonizing antibodies in patients with this syndrome.4 Controversy exists regarding the value of serum muramidase values for reflecting myeloid cell turnover.28 In our patients, these values were normal or low in 28 of 30 individuals tested, providing no evidence for increased myeloid cell turnover in this syndrome.

These studies indicate that patients with CINS may be subclassified into groups according to their neutrophil levels. All subjects had normal or decreased levels of CSA in marrow, which may have contributed to defective myeloid maturation as a major cause for the neutrope-

myeloid cell turnover in this syndrome.

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